The first ATPase domain of the yeast 246-kDa protein is required for in vivo unwinding of the U4/U6 duplex

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ABSTRACT

The yeast *PRP44* gene, alternatively named as *BRR2*, *SLT22*, *RSS1*, or *SNU246*, encodes a 246-kDa protein with putative RNA helicase function during pre-mRNA splicing. The protein is a typical DEAD/H family member, but unlike most other members of this family, it contains two putative RNA helicase domains, each with a highly conserved ATPase motif. Prior to this study little was known about functional roles for these two domains. We present genetic and biochemical evidence that ATPase motifs of only the first helicase domain are required for cell viability and pre-mRNA splicing. Overexpression of mutations in the first domain results in a dominant negative phenotype, and extracts from these mutant strains inhibit in vitro pre-mRNA splicing. In vitro analyses of affinity purified proteins revealed that only the first helicase domain possesses poly (U)-dependent ATPase activity. Overexpression of a dominant negative protein in vivo reduces the relative abundance of free U4 and U6 snRNA with a concomitant accumulation of the U4/U6 duplex. Accumulation of the U4/U6 duplex was relieved by overexpression of wild-type Prp44p. Three DEAD/H box proteins, Prp16p, Prp22p and Prp44p, have previously been shown to affect U4/U6 unwinding activity in vitro. The possible role of these proteins in mediating this reaction in vivo was explored following induced expression of ATPase domain mutants in each of these. Although overexpression of the mutant form of either Prp16p, Prp22p, or Prp44p was lethal, only expression of the mutant Prp44p resulted in accumulation of the U4/U6 helix. Our results, when combined with previously published in vitro results, support a direct role for Prp44p in unwinding of the U4/U6 helix.

Keywords: pre-mRNA splicing; RNA helicase; *Saccharomyces cerevisiae*; U4/U6 unwinding in vivo

INTRODUCTION

Nuclear pre-mRNA splicing occurs via two successive transesterification reactions. In the first, the 2' hydroxyl of the branch-point adenosine attacks the 5' exon/intron boundary, forming the splicing intermediates exon I and lariat-exon 2. The second reaction involves a nucleophilic attack by the 3' hydroxyl of exon 1 at the 3' splice site, resulting in ligated exons 1 and 2 and a free intron in the form of lariat. These reactions occur in the spliceosome, which harbors U1, U2, U4, U5, and U6 snRNPs along with numerous non-snRNP splicing protein factors. In the process of spliceosome formation and the splicing reactions, there are a series of sequential and dynamic RNA interactions among the

snRNAs and between the snRNAs and pre-mRNA (for review, see Green, 1991; Guthrie, 1991; Moore et al., 1993; Will & Lührmann, 1997; Staley & Guthrie, 1998). After joining of the U4/U6, U5 tri snRNPs to the spliceosome, the highly conserved U4/U6 duplex is dissociated and U6 snRNA forms base pairing interactions with U2 snRNA and the 5' splice site (Madhani & Guthrie, 1994; Ares & Weiser, 1995). It is believed that the ATP-dependent RNA helicases of the DEAD/DEXH box family play a major role in rearrangements of the spliceosome (Schwer & Guthrie, 1992a; Kim & Lin, 1993; Fuller-Pace, 1994; Xu et al., 1996; Yean & Lin, 1996; Staley & Guthrie, 1998).

The DEAD/DEXH-box proteins are involved in diverse cellular functions including pre-mRNA splicing (Wassarman & Steitz, 1991; Ripmaster et al., 1992; Schmid & Linder, 1992; Czaplinski et al., 1995; Liang et al., 1996; Margossian et al., 1996; Ohno & Shimura, 1996; Py et al., 1996; Chuang et al., 1997). This family

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is characterized by several highly conserved ATPase motifs including the DEAD or DExH sequence (Chang et al., 1990; Koonin, 1991; Bork & Koonin, 1993). Functional assignments for several of the motifs have been made through analyses of various RNA helicases, including the extensively studied *eIF4A* (Pause & Sonenberg, 1992; Pause et al., 1993, 1994). Mutations in ATPase A motif (GKT, motif I) affect ATP binding, whereas mutations in ATPase motif B (DEAD or DExH, motif II) affect ATP hydrolysis. Motif VI (GRAGR) is involved in ATP hydrolysis, and the SAT sequence element (motif III) is essential for RNA unwinding. Mutational alterations in these motifs result in specific functional impairment of each helicase thus far studied (Ohno & Simura, 1996; Burgess & Guthrie, 1993; Plumpton et al., 1994).

Analysis of dominant negative mutations has been proposed as a tool for identifying the function of a gene product by observing the functional and biochemical consequences of overexpressing mutant proteins in the presence of the wild-type protein (Herskowitz, 1987). It has been suggested that the generation of dominant negative mutations that affect the ATPase domains of DEAD or DExH proteins could provide a powerful strategy for elucidating the diverse roles of this gene/protein family (Schwer & Guthrie, 1992b). Several dominant negative mutants in ATPase domains have been created to investigate the function of DEAD/H-box proteins (Schwer & Guthrie, 1992b; Burgess & Guthrie, 1993; Plumpton et al., 1994; Ohno & Simura, 1996; Kim & Lin, 1996). Studies of mutations in pre-mRNA splicing factors *PRP2* and *PRP16* have shown that mutant proteins with reduced ATPase activity remain tightly bound to their spliceosomal substrate (Schwer & Guthrie, 1992b; Plumpton et al., 1994; S.H. Kim & R.J. Lin, unpubl. data). Thus, reduced ATPase activity prevents dissociation of the mutant proteins from their substrates.

A novel DExH family protein that is a putative RNA helicase in yeast has been independently identified in four different laboratories via various screens (Lauber et al., 1996; Lin & Rossi, 1996; Noble & Guthrie, 1996; Xu et al., 1996). This protein is 2,146 amino acids in length and contains two putative RNA helicase domains (Lauber et al., 1996; Xu et al., 1996). We have identified this gene by a screen for suppressors that rescued a block to splicing created by an experimentally introduced structure in exon 2 of a yeast *ACT1-LACZ* fusion construct (Lin & Rossi, 1996). A temperature-sensitive suppressor with a single amino acid change close to motif VI of the first helicase domain was identified in a previously uncharacterized open reading frame located in chromosome V. We designated this gene as *RSS1* (Restores Splicing and Stability). In addition to the suppression of the block to step 2 in splicing of the experimental construct, the mutant protein resulted in a decrease in the steady-

state levels of *ACT1* and *CYH2* messages at the non-permissive temperature. A mutation in the same gene designated as *BRR2* (Bad Response to Refrigeration) was identified among a collection of cold-sensitive mutants that exhibited diminished splicing at low temperature (Noble & Guthrie, 1996). It has been shown that the coimmunopurified Brr2p can unwind the U4/U6 duplex in an ATP-dependent manner in vitro (Raghu-nathan & Guthrie, 1998a). In the absence of added pre-mRNA, coimmunopurified Brr2p dissociates the U4/U6 duplex and releases free U4 and U6. The U4/U6 duplex accumulates in a cold-sensitive mutant extract, further implicating that Brr2p is involved in destabilizing or unwinding the U4/U6 duplex in vitro. An antagonistic regulation of U4/U6 association–dissociation by *PRP24* and *BRR2* has been suggested based upon in vitro analyses: Brr2p directs ATP-dependent dissociation of U4/U6 snRNPs, whereas Prp24p restores the disrupted duplex (Raghu-nathan & Guthrie, 1998b). The same gene (designated *SLT22*) has been identified in a screen for synthetic lethality in combination with a nonlethal mutation of U2 snRNA (Xu et al., 1996). The purified protein encoded by *SLT22* was shown to have U2/U6 snRNA-dependent ATPase activity and an essential role in pre-mRNA splicing. The fourth independent isolation came from a biochemical analysis of a HeLa 200-kDa U5 snRNA-associated DExH protein that was shown to be homologous to the yeast 246-kDa species, and the corresponding yeast protein was labeled as *SNU246* (Lauber et al., 1996). It was shown by antibody inhibition that the U5–200-kDa protein is involved in step 2 of pre-mRNA splicing. It has also been demonstrated that purified U5 snRNP harboring the 200-kDa protein stimulates unwinding of in vitro synthesized U4/U6 duplex as well as a nonspecific RNA duplex in vitro (Laggerbauer et al., 1998).

To clarify potential confusion generated by the use of multiple names for the same gene/protein, we recommend that the gene encoding the 246-kDa protein be designated *PRP44* in accordance with the nomenclature for splicing-associated factors in yeast. Thus, throughout the remainder of this text, we will refer to the gene as *PRP44* and the protein as Prp44p.

During active spliceosome formation the pairing between U4 and U6 is disrupted and establishment of new pairings between U6/U2 and U6 and partial replacement of the U1 pairing site at the 5' splice site take place (Murray & Jarrell, 1999; Staley & Guthrie, 1999). Unwinding of the U4/U6 duplex triggers these new interactions and converts the splicing complex from an inactive to an active state. Since the helicase(s) involved in U4/U6 unwinding should have stringent substrate recognition properties for this important process, it is somewhat surprising that at least three DEAD/H proteins (Prp44p, Prp16p, and Prp22p) have been shown to effect this unwinding in vitro (Laggerbauer et al., 1998; Schwer & Gross, 1998; Wagner et al.,

1998; Wang et al., 1998). Prior to this study, no evidence for the in vivo activities of these proteins on the U4/U6 unwinding reaction has been determined.

Both the yeast 246-kDa and Hela 200-kDa proteins possess two putative RNA helicase domains, each of which could be essential for the helicase function(s) of this protein (Lauber et al., 1996; Xu et al., 1996). It was suggested that the two domains might functionally behave as a dimer, as was observed for an oligomeric DNA helicase (Staley & Guthrie, 1998). We have investigated the role of the two putative helicase domains of *PRP44* by a site-directed mutagenesis approach. The highly conserved ATPase motifs were independently mutagenized and assayed for in vitro and in vivo function. We show that only the first domain possesses an independently functional ATPase activity. Overexpression of a mutant in the first helicase domain results in a dominant negative phenotype. Analyses of U4 and U6 RNAs from cells overexpressing the mutant alleles show a marked accumulation of U4/U6 duplex with a reduction in the free U4 and U6 species in vivo. In contrast, overexpression of helicase ATPase domain mutations in Prp16p and Prp22p domain had no effect on the U4/U6 complex. Thus, in conjunction with previously published data based upon in vitro analyses, the results presented here strongly support a direct role for a U4/U6 unwinding function of *PRP44* in vivo. We also demonstrate the role of the first RNA helicase domain in this activity.

RESULTS

Motifs I and II of helicase domain I, but not domain II, are essential for cell viability

As tools for investigating the in vivo function of *PRP44*, we created two sets of mutations. Figure 1 depicts the two putative helicase domains of *PRP44* with motifs conserved among the DExH family. This figure also illustrates the four mutants that were constructed in these domains. The genetic changes were made based upon mutational analyses of other RNA helicases in which similar alterations resulted in dominant negative phenotypes (Pause & Sonenberg, 1992; Burgess & Guthrie, 1993; Ohno & Simura, 1996; S.H. Kim & R.J. Lin, unpubl. data). Following site-directed mutagenesis, the altered genes were sequenced to confirm the changes.

Mutant alleles in a LEU^+ vector were transformed into a *PRP44* knockout strain (see Materials and Methods for details), harboring a plasmid-borne copy of wild-type *PRP44* in the vector YCp50. URA^+ , LEU^+ transformants were streaked onto 5-FOA plates lacking histidine for selective elimination of the wild-type gene in the YCp50 vector (Fig. 2A). Under these selective conditions, there were no colonies arising from transformants with the domain I mutations. These re-

FIGURE 1. Primary structure of the *PRP44* gene. The two putative RNA helicase-like domains with highly conserved ATPase motifs GKT, DExH, SAT, and GRAGR are indicated. Using site-directed mutagenesis, GKT was mutated to DNT and DExH to GExH. These genetically altered motifs are labeled as I-1 and I-2 for domain I, and II-1 and II-2 for the second domain. The relative positions for two *EcoRI* sites and an *NcoI* site for creation of the *PRP44* knockout strain are indicated.

sults suggested that the GKT and DExH regions of domain I, but not domain II, are essential for *PRP44* function and cell viability.

Overexpression of *PRP44* with domain I mutations causes a dominant negative phenotype in vivo and in vitro

To test whether the domain I or II mutations conferred a dominant negative phenotype, the mutants were

FIGURE 2. The first helicase domain of Prp44p is essential for cell viability and can be mutated to create a dominant negative allele. **A:** Wild-type or mutant *PRP44* genes, under control of the *CUP1* promoter, were transformed into the *PRP44* knockout strain. Transformants were plated on 5-FOA plates to select for loss of the YCp50 vector-borne wild-type *PRP44* gene as described in Materials and Methods. The helicase motif mutations in domain I are marked as I-1 or I-2 and the second motif mutations as II-1 or II-2. **B,C:** The wild-type or mutant versions of *PRP44* under control of the *CUP1* promoter were transformed into wild-type yeast cells. The resulting URA^+ transformants were streaked onto uracil-deficient plates with no added Cu_2^+ (**B**) or onto the same plate containing 1 mM Cu_2^+ (**C**).

cloned under the inducible *CUP1* promoter. Copper-induced expression of these mutant proteins should result in elevated intracellular levels relative to the wild-type protein, which is under the control of its own promoter (Trotta et al., 1997). The wild-type or mutant genes in the *CUP1* promoter were transformed into a wild-type yeast cell (Fig. 2B,C). In the absence of copper, a slight growth defect of strains harboring the domain I mutations was observed, but cells maintained their viability (Fig. 2B and data not shown). Expression of the mutant proteins was induced with varying concentrations of CuSO_4 ranging from 0.1 mM to 1 mM (Fig. 2C and data not shown). At 1 mM CuSO_4 , there were significant differences in the growth of the domain I mutants compared with the control cultures in which no copper was added (Fig. 2B,C). Neither of the yeast strains harboring domain I mutants grew in the presence of CuSO_4 . Strains harboring the domain II mutants or wild-type *PRP44* exhibited no growth differences in the absence or presence of up to 2.5 mM CuSO_4 . We therefore conclude that overexpression of domain I mutant proteins results in a dominant negative phenotype in the presence of wild-type Prp44p.

To verify that the levels of expression of the various Prp44p were similar, the levels of Prp44p after copper induction were monitored by Western blotting (data not shown). No significant differences in expression between wild-type and the mutants were detected, indicating that the dominant negative phenotype is caused by a nature of proteins instead of protein stability or expression level difference.

We next sought to determine if the dominant negative phenotype is connected to a specific pre-mRNA splicing defect in vitro. The wild-type and several mutant alleles were cloned under the *GAL1* promoter and transformed into the wild-type cell for another induction experiment. Glass-bead extracts were prepared from cells with and without galactose induction, and these extracts were mixed with wild-type splicing extracts and incubated on ice for 15 min. The preincubated extract mixtures were then incubated under splicing conditions at 23 °C for 30 min with a ^{32}P -labeled *ACT1* pre-mRNA splicing substrate. Extracts prepared from strains expressing wild-type *PRP44* or mutants in domain II had no inhibitory effects on splicing. In contrast pre-mRNA splicing was completely blocked at or prior to step 1 by addition of extracts prepared from copper-induced domain I mutants (Fig. 3, lanes 7 and 8). To rule out any indirect effects of added extract, the in vitro inhibition experiments were also carried out with purified Prp44p proteins (Fig. 3, lanes 11–16). Ten nanograms of each purified protein were mixed with the wild-type splicing extract, and splicing activity was monitored. Consistent with the glass-bead extract results, there was an inhibition of activity by addition of the Prp44p with the first domain mutations. The in vitro splicing inhibition results correlate with the in vivo data demonstrating that

FIGURE 3. Prp44p prepared from dominant negative mutants inhibits in vitro splicing activity. Wild-type splicing extracts were mixed with glass-bead extracts prepared from yeast strains transformed with the various mutant alleles of *PRP44* under the *GAL1* promoter without (the first set of experiments) or with (second set) galactose induction. These were then incubated for 15 min on ice followed by addition of splicing reaction reagents including ATP and ^{32}P -labeled *ACT1* pre-mRNA substrate. The splicing reactions were performed at room temperature for 30 min followed by extraction with phenol. The RNAs were resolved on a 7.5% denaturing polyacrylamide gel (Kim & Lin, 1993). The splicing inhibition activity was also tested using 10 ng of purified proteins (third set).

domain I, but not domain II, is required for pre-mRNA splicing and domain I mutant proteins exhibit a dominant negative phenotype when added to splicing extracts.

Dominant negative *PRP44* proteins lack ATPase activity

The wild-type and domain I and II mutant alleles of *PRP44* were fused to His and Flag tags under the control of the *CUP1* promoter to facilitate purification of the proteins from cellular extracts. Testing of the tagged wild-type protein in an *PRP44* knockout background revealed no alterations in protein function as a consequence of the expressed tag epitopes. Mutant and wild-type proteins were sequentially purified through a Ni-column followed by coimmunoprecipitation with anti-Flag antibody (see Materials and Methods). The purity and amount of the proteins after Ni-column and coimmunoprecipitation were analyzed by SDS-PAGE and silver staining (Fig. 4A). The purified proteins were used in poly-(U) RNA-dependent ATPase assays as previously described (Kim & Lin, 1993). Baseline ATPase activity was detected in the absence of poly-(U) RNA, whereas upon addition of poly-(U) RNA, about fivefold

FIGURE 4. Only the first helicase domain of Prp44p is functional as an ATPase. **A:** A *PRP44* knockout strain was transformed with *PRP44* mutant alleles fused to 5' His and Flag tag sequences (Trotta et al., 1997). Two liters of cell culture were used to prepare the splicing extracts by the liquid nitrogen method (Umen & Guthrie, 1995). Prp44p was purified using a two-step procedure, which includes a nickel column and coimmunoprecipitation with an anti-Flag tag antibody (see Materials and Methods). **A:** Five micrograms of each purified protein were analyzed by SDS-PAGE and stained by silver staining. **B:** Twenty micrograms of each protein were used for assaying ATPase function in the presence or absence of a poly(U) RNA substrate (Kim & Lin, 1993). The relative amounts of ATPase activity were determined by dividing the ATPase cpm obtained from each of the mutant proteins with the amount obtained from the wild-type protein. Each bar graph represents the average of two experimental determinations.

enhancement of ATPase activity was observed with wild-type and domain II mutant proteins. In contrast, domain I mutant proteins had less than 20% of wild-type ATPase activity even in presence of poly-(U) RNA (Fig. 4B). Mock-purified protein from untagged wild-type extracts served as both negative and background controls for these experiments. We conclude from these and other results that the Prp44p is an RNA-dependent ATPase and only domain I is required for ATPase activity.

Domain I is required for unwinding of the U4/U6 duplex *in vivo*

The human homolog of Prp44p is the HeLa U5-associated 200-kDa protein. It has been shown that this protein can mediate unwinding of U4/U6 *in vitro* (Laggerbauer et al., 1998). Furthermore, ATP-dependent *in vitro* unwinding of the U4/U6 duplex has been observed using partially purified Prp44p (Brr2p) (Ragunathan & Guthrie, 1998a). The mutant allele (*brr2-1*) also maps to the

highly conserved ATPase domain I, and fails to mediate U4/U6 unwinding in vitro. Based upon these in vitro observations, we next sought to determine whether our dominant negative domain I mutants affected the U4/U6 RNA unwinding in vivo. To investigate this possibility, we utilized a native gel, RNA-annealing approach first described by Li and Brow (1993). Total RNAs were prepared from *PRP44* wild-type cells containing plasmid-borne wild-type or mutant *PRP44* genes under the

inducible promoter, *CUP1*. The RNAs were prepared with or without copper induction of Prp44p. The levels of free and duplex forms of U4 and U6 were analyzed following annealing to 32 P-labeled U6 or U4 complementary oligos. The complexes were analyzed by autoradiography following electrophoresis in a nondenaturing polyacrylamide gel (Fig. 5A).

RNA-oligo hybrids prepared from strains with wild-type or domain II mutants showed virtually identical U4,

FIGURE 5. Dominant negative mutants of *PRP44* are defective in dissociating U4/U6 duplexes. Total cellular RNA was prepared from strains transformed with the various *PRP44* mutant alleles. RNAs were prepared from these strains either with or without Cu₂⁺ induction. **A:** Total cellular RNAs from the indicated strains were prepared with or without Cu₂⁺ induction and hybridized to oligonucleotides complementary to U4 or U6 snRNAs. The hybridized products were electrophoresed in a nondenaturing polyacrylamide gel followed by autoradiography (Li & Brow, 1993). The U4/U6 complex as well as uncomplexed U4 and U6 are indicated. **B:** The same amount of RNA samples of **A** was heated at 70 °C for 10 min to destroy any U4/U6 duplex and hybridized with 32 P-labeled U1, U2, U4, U5, and U6 primers to compare total amount of snRNAs in each sample. **C:** Quantitation of the total amount of U6 and U4 snRNAs. The amounts of U4 and U6 in duplex or single form from **A** were standardized with the total amounts of U4 and U6 snRNAs in **B**. The relative percentages of U4/U6 duplex and free U4 and U6 from the nondenaturing gels of **A** versus total U4 and U6 snRNAs from gel of **B** were analyzed and quantified on an AMBIS4000 radioanalytic image system. **D:** The wild-type, I-1, and I-2 mutant genes were cloned under the *GAL1* promoter, and transformed into wild-type cells. Total RNA was prepared before and after galactose induction, hybridized with the U5 and U6 probes, and analyzed on a native gel as described in **A**. In contrast to the *CUP1* promoter experiments, there was no significant U4/U6 duplex accumulation without induction of the mutant proteins.

U6, and U4/U6 patterns with and without copper induction. In contrast, domain I mutants strongly affected the ratio of U4/U6 duplex to free U4 and U6 snRNAs. [The slight increase in the U4/U6 duplex after copper induction in the case of the II-1 mutant allele is most likely due to unequal sample loading (Fig. 5A, lane 8)]. In the domain I mutants, U4/U6 duplexes accumulate relative to free U4 and U6 even before copper induction due to leakiness in the *CUP1* system by contaminated copper in media. This observation coincides with the slow grow phenotypes of mutant 1-1 and 1-2 on plates containing no added copper.

To compare the total amounts of U4 and U6 RNAs in each sample, the same amounts of RNA samples as depicted in Figure 5A were heated at 70°C for 10 min to destabilize the U4/U6 duplex. The unduplexed forms of these RNAs were hybridized with the probes and loaded onto a native gel (Fig. 5B). Comparisons of Figures 5A and 5B demonstrate that the total amounts of U4 and U6 snRNAs are virtually identical in each sample, and therefore only the amount of these snRNAs in the form of a duplex differs between domain I mutant RNA preparations and the wild-type and domain II mutant preparations. Because Prp44p is a U5 snRNP component and a domain I mutation has been reported to cause accumulation of a complex lacking U5 snRNA (Xu et al., 1996), the levels of U5 along with U1 and U2 snRNAs were also examined. (Fig. 5B). The amounts of these other snRNAs were similar in the different RNA preparations.

The autoradiographic signals were quantitated on an AMBIS radioanalytic imager (Fig. 5C). The data represent the relative ratios of the duplex versus free U4 and U6 RNA from the experiments presented in Figure 5A after normalization to the total amount of U4 and U6 snRNAs from the results depicted in Figure 5B. These data demonstrate that the total amounts of U4 and U6 snRNAs are the same in each RNA preparation and only the ratio of the duplex to free forms varies when the dominant negative mutants of Prp44p are copper induced. These experiments further suggest that the first ATPase domain of Prp44p is required for unwinding the U4/U6 duplex.

To analyze the connection between induction of mutant Prp44p and accumulation of the U4/U6 duplex more carefully, different *PRP44* alleles were placed under the transcriptional control of the more tightly regulated *GAL1* promoter system. *PRP44* and two dominant negative mutations were cloned under the *GAL1* promoter and transformed into wild-type cells. Galactose induction of the dominant negative alleles, but not wild-type *PRP44*, resulted in growth cessation (data not shown). Total cellular RNA was prepared from both induced and noninduced transformants, and the U4/U6 snRNA patterns were analyzed using the native gel assay (Fig. 5D). Without galactose induction of the domain I mutant proteins, there is no variation in the du-

plex versus free U6 ratios as compared to wild-type Prp44p (Fig. 5D, lanes 1, 2, 3, and 5). Galactose induction of the mutant proteins resulted in a marked accumulation of the U4/U6 with a corresponding decrease of free U6 snRNA (Fig. 5D, lanes 4 and 6). As a loading control of total RNA amount, the two different forms of U5 snRNAs were monitored by U5 snRNA specific primer. Thus, these results provide independent confirmation of the *CUP1* promoter results, and illustrate that the small levels of duplex accumulation without induction of *CUP1* promoter were due to leakiness in this expression system.

Because an accumulation of the U4/U6 duplex was observed with overexpression of *PRP44* mutant proteins, we asked if we could rescue this accumulation with elevated expression of the wild-type Prp44p. Wild-type cells were transformed with the following vectors. The first vector harbored either a dominant negative mutant or wild-type *PRP44* under transcriptional control of the *CUP1* promoter. The other vector contained the wild-type *PRP44* coding region under control of the *GAL1* promoter (Fig. 6A). In this system, expression of Prp44p (mutant or wild-type) depends on addition of the appropriate inducer, copper or galactose. Following cell growth under different induction conditions, RNA samples were prepared and analyzed as described in the legend to Figure 5B (Fig. 6B). As controls, RNA samples were prepared from wild-type cells propagated under the same three conditions described above. Induction of wild-type Prp44p had no effect on the ratio of duplexed U4/U6 to the free forms (Fig. 6B, lanes 1, 2, and 3). Induction of the domain I dominant negative mutant resulted in the expected accumulation of U4/U6 duplex which was rescued following galactose induction of wild-type Prp44p (Fig. 6B, lanes 4, 5, and 6). These results provide additional evidence for the involvement of Prp44p in mediating the in vivo dissociation of the U4/U6 duplex.

DNT mutation in Prp44p, but not Prp16p and Prp22p, inhibits U4/U6 unwinding in vivo

The DEAD/H-box proteins Prp16p and Prp22p have both been shown to have NTP-hydrolysis dependent U4/U6 unwinding in vitro (Wagner et al., 1998; Wang et al., 1998). To determine whether or not our observations on the inhibitory effect of the DNT mutants in Prp44p on U4/U6 unwinding in vivo were unique to this protein, we created the same mutations in the ATPase domains of *PRP16* and *PRP22*. The DNT mutations in the ATPase domains were introduced into the *PRP16* and *PRP22* genes by site-directed mutagenesis. These mutant alleles were cloned under the *GAL1* promoter and transformed into wild-type cells. These transformants were viable in noninducing glucose media, but nonviable in the presence of inducing levels of galac-

FIGURE 6. Prp44p dissociates U4/U6 duplexes in vivo. **A:** Diagrammatic explanation of the different induction conditions used for preparation of each RNA sample. Wild-type cells were cotransformed with plasmids pYES2.0-*PRP44* and pDK1-WT or pDK1-dn1. pYES2.0-*PRP44* expresses Prp44p under control of the *GAL1* promoter of the pYES2.0 vector. The vectors pDK1-WT or pDK1-dn1 contain wild-type *PRP44* or the domain I-1 mutant under transcriptional control of the *CUP1* promoter. The first RNA sample was prepared from cells grown in glucose media without exogenous copper ion addition. The second RNA sample was prepared following treatment of the first sample cells with 5 mM CuSO_4 for 4 h. The third sample was prepared from the second sample following induction of wild-type Prp44p with galactose. Following incubation under one growth condition, cells were washed with distilled water and resuspended under the induction conditions. **B:** Total cellular RNAs were prepared from cells cultivated as described in **A**, and analyzed by hybridizing to the U4 and U6 primers followed by nondenaturing gel electrophoresis (Li & Brow, 1993).

tose (Fig. 7). These data support the idea that the DNT change in highly conserved ATPase domains of DEAD/H-box proteins can result in dominant negative phenotypes following overexpression.

To examine the effects of overexpression of the *Prp16* and *Prp22* mutant alleles on U4/U6 unwinding, the patterns of U4/U6 and free U6 from noninduced versus

galactose-induced cultures were compared to a Prp44p domain 1 mutant (Fig. 8). As observed before, there is an accumulation of the U4/U6 duplex with induction of the mutant allele of *PRP44* (Fig. 8, lane 2). In contrast, no accumulation of this duplex occurs following overexpression of the mutant forms of Prp16p and Prp22p (Fig. 8, lanes 3, 4, 5, and 6). Therefore, despite in vitro results demonstrating potential involvement of Prp16p and Prp22p in unwinding of the U4/U6 helix, ATPase domain mutants of these proteins do not appear to affect this reaction in vivo. Among the DEAD/H box helicases that mediate U4/U6 duplex unwinding activity in vitro, Prp44p appears to be unique in its functional role in this process in vivo. Correlation of the in vitro and in vivo data involving Prp44p supports an essential role for this protein in unwinding of the U4/U6 duplex.

DISCUSSION

This study provides the first direct evidence that Prp44p (Brr2p, Slit22p, Snu 246p, and Rss1p) affects dissociation of U4/U6 RNA duplexes in vivo. We have explored the intracellular function of wild-type Prp44p along with dominant negative mutants by taking advantage of the inducible expression conferred by the *CUP1* or *GAL1* promoters. The effects of induction were monitored by

FIGURE 7. Overexpression of ATPase domain mutants in *Prp16* and *Prp22* render cells a dominant negative phenotype. Wild-type cells were transformed with either empty vector or DNT mutants of *Prp44*, *Prp16*, or *Prp22*. **A:** On glucose media there are no detectable inhibitory effects. **B:** All three DEAD/H mutant clones affect cell viability with galactose induction.

FIGURE 8. The DNT mutants of Prp44p, but not Prp16p and Prp22p mutants, inhibit U4/U6 unwinding following induction in vivo. Total RNAs were prepared from noninduced and induced cultures harboring the genes of interest. Accumulation of U4/U6 duplex was monitored as described in the legend to Figure 5.

using a nondenaturing gel assay to detect U4/U6 duplexes and free form RNAs under varying conditions of wild-type or dominant negative Prp44p expression. Induced expression of ATPase domain I mutants resulted in an accumulation of U4/U6 duplexes, but no net loss or gain in the total amount of snRNAs (Fig. 5B,D). We reasoned that if Prp44p is a mediator of U4/U6 dissociation in vivo, induced, elevated expression of wild-type Prp44p in a dominant negative mutant background should prevent and/or relieve accumulation of the U4/U6 duplex. In fact this is the observed result (Fig. 6), providing additional evidence that Prp44p is such a mediator. We have also demonstrated that, of the two putative helicase domains in Prp44p, only mutagenic alteration of the first domain affects ATP hydrolysis and consequent U4/U6 duplex stability. Mutants in Prp44p domain II had no observable effects on ATPase and U4/U6 stability. The functional role of domain II, if any, still remains unanswered.

Other groups have conducted in vitro experiments to study the potential RNA unwinding activities of DEXH/D proteins (Laggerbauer et al., 1998; Raghunathan & Guthrie, 1998a; Schwer & Gross, 1998; Wagner et al., 1998; Wang et al., 1998). The highly purified human homolog of Prp44p, HeLa derived U5–200kDp, has been shown to mediate unwinding of in vitro synthesized U4/U6 (Laggerbauer et al., 1998). However, the U5–200kDp also unwinds another unrelated RNA duplex in vitro. Similar in vitro U4/U6 unwinding results have been obtained with the yeast 246-kDa protein

(Raghunathan & Guthrie, 1998a). The mutant *brr2-1* (mutant in the first helicase domain) was incapable of supporting this unwinding reaction in vitro. Taken together, these in vitro data strongly support a role for Prp44p in destabilizing or unwinding of the U4/U6 duplex, but to date such a role has not been demonstrated in vivo.

It should be emphasized that in vitro helicase or unwinding activities are not necessarily indicative of in vivo function. For example, in vitro unwinding of U4/U6 occurs in the absence of added pre-mRNA substrates in a nonsplicing coupled reaction (Raghunathan & Guthrie, 1998a). Furthermore, in vitro unwinding liberates free U4 and U6; but in a splicing reaction, this liberation is tightly coupled with new interactions of U6 with both U2 and the pre-mRNA (Madhani & Guthrie, 1994; Staley & Guthrie, 1998). We have in fact observed that Prp44p is also required for stabilization of free U6 following U4/U6 duplex unwinding (D.-H. Kim & J.J. Rossi, unpubl. data). It is noteworthy that splicing extracts prepared from the cold-sensitive *brr2-1* mutant of *PRP44* contained low levels of the U4/U6, U5 tri-snRNP along with a diminished amount of free U5 and U2 snRNAs (Raghunathan & Guthrie, 1998a). In addition, it was observed that extracts from this mutant harbor a slowly migrating complex that hybridizes to U4, U6, U5, and U2 probes, suggesting that, apart from the defect in helicase activity, the mutant protein may be altered in its ability to associate with other components of the spliceosome. We have observed that extracts from our dominant negative mutant strains inhibit spliceosome formation (data not shown), suggesting a functional role for Prp44p in spliceosome assembly. Unwinding of U4/U6 may be required for completion of spliceosome assembly. Alternatively, but not mutually exclusive, the ATPase activity of Prp44p may be required for some earlier step in spliceosome assembly. Both of these possibilities are currently under investigation.

Several studies have demonstrated that mutagenic alterations of the highly conserved helicase motifs found in DEXH/DEAD box proteins generate dominant negative phenotypes (Ohno & Simura, 1996; Schwer & Guthrie, 1992b; Burgess & Guthrie, 1993; Plumpton et al., 1994; S.H. Kim & R.J. Lin, unpubl. data). An extensive mutational analysis of *eIF4A* demonstrated that the GKT and DEXH/D regions are required for the ATP binding and ATP hydrolysis, respectively (Pause & Sonenberg, 1992; Pause et al., 1993). Both mutations also result in defects in RNA unwinding activity. In our study, the analogous alterations in Prp44p domain I, but not domain II, also resulted in defective ATPase and RNA unwinding activities (Figs. 4B, 5A,D). Results from other studies have led to postulations that the DEXH/D protein family requires the GKT sequence for ATP binding and the DEXH/D sequence for ATP hydrolysis. Mutations in either sequence result in RNA unwinding defects. We have demonstrated that mutations

in both of these regions create dominant negative proteins that inhibit U4/U6 duplex unwinding in vivo. The experimental approaches used in this study to elucidate Prp44p function in vivo should be applicable to studies of other putative helicases involved in various steps of pre-mRNA splicing.

Our results have established that in vivo unwinding of U4/U6 duplex requires an active ATPase activity of Prp44p. These experiments provide direct links between ATPase function, RNA unwinding and pre-mRNA splicing. The overall consequence of ATP hydrolysis by Prp44p is the same as that of other DExH proteins such as Prp2p, Prp5p, Prp16p, Prp22p, Prp28p, and Prp43p, all of which may be involved in ATP-dependent RNA rearrangements (Schwer & Guthrie, 1992a; O'Day et al., 1996; Yean & Lin, 1996; Arenas & Abelson, 1997; Wagner et al., 1998; Staley & Guthrie, 1999). Since these proteins all share extensive homologies within the helicase motif (Chen & Lin, 1990; Company et al., 1991; Schwer & Guthrie, 1992a; O'Day et al., 1996; Arenas & Abelson, 1997; Staley & Guthrie, 1999), it is likely they also catalyze or mediate RNA unwinding activities. The DExH box splicing factors Prp16p and Prp22p have been shown to destabilize in vitro-synthesized U4/U6 duplexes (Schwer & Gross, 1998; Wagner et al., 1998; Wang et al., 1998). We asked whether these proteins are also involved in U4/U6 duplex unwinding in vivo by using a mutational approach analogous to that used for Prp44p (Fig. 8). Our results suggest that the U4/U6 duplex unwinding activity of Prp16p and Prp22p observed in vitro may not be relevant in vivo. It is highly likely that Prp44p interacts with other spliceosomal factors, such as Prp38p to mediate U4/U6 dissociation (Xie et al., 1998). Further elucidation and functional characterization of spliceosomal factors that interact with Prp44p in the U4/U6 unwinding reaction will facilitate our understanding of this critically important step in pre-mRNA splicing.

MATERIALS AND METHODS

Yeast methods and strains

Yeast genetic manipulations were performed using standard methods (Guthrie & Fink, 1991). For the plasmid shuffle assay, a *PRP44* knockout strain was created as follows: The 3.5-kb *EcoRI* fragment from *PRP44* was cloned into the *EcoRI* site of the vector pRS303 (Sikorski & Heiter, 1989, and Fig. 1). The vector containing the *PRP44* fragment was linearized by *NcoI* digestion and used to transform the diploid strain YS2 harboring the entire wild-type *PRP44* gene in the yeast vector YCp50. *URA*⁺, *HIS*⁺ transformants were selected and allowed to sporulate for tetrad dissection. Among the tetrads, the expected phenotype for the *PRP44* knockouts is *URA*⁺, 5-*FOA* sensitive and *HIS*⁺. One such knockout strain, designated as KN1, was selected and used for the plasmid shuffle assays. Yeast strains used in this study are as follows:

YS2 (diploid resulting from a mating of 1827 and 1826): *MATa/α, ade2-101/ade2-101, his3-Δ200/his3-Δ200, ura3-52/ura3-52, leu2-3/LEU2, PRP44:HIS3/PRP44*
 KN1 (RSS1 knockout strain): *MATa, ade2-101, his3-Δ200, ura3-52, leu2-3, PRP44:HIS3*
 B4-7-2D (a wild-type cell for extract): *MAT a, his3-Δ200, ura3-52, lys1*
 1827: *MATα, ade2-101, his3-Δ200, leu2-3, ura3-52*
 1826: *MATa, ade2-101, his3-Δ200, ura3-52*
 B4-7-2D: *MATa, his3Δ200, URA3-52, lys1*
 PRP16-1: *MATa ade2-101, his3Δ200, tyr1, URA3-52* (Vijayraghavan et al., 1989)
 PRP22-1: *MATa ade2-101, his3Δ200, tyr1, URA3-52* (Vijayraghavan et al., 1989).

Plasmid construction and in vitro mutagenesis

The *CUP1* promoter, His tag, Flag tag, and *ADH1* terminator derived from YpH8/FLAG (Trotta et al., 1997) were introduced into the *HindIII* and *SacI* sites of the pRS315 vector (Sikorski & Heiter, 1989). The new vector was designated as pDK101. Two primers (RSS12: 5'-AATCCATTTCGAGGATCC AGGACTAAACA-3' and RSS7: 5'-AATCCATTTCGAGGATC CAGGACTAAACA-3') were used to clone the *PRP44* gene following amplification with the *Pfu* polymerase system from Stratagene (La Jolla, California). The PCR products were gel purified, digested with *BclI*, and ligated into *BamHI*-digested pDK101. To test whether or not *PRP44* prepared by this method is functional, the pDK101-*PRP44* plasmid was transformed into a strain harboring a temperature-sensitive *PRP44* allele and assayed for growth at 36 °C. Additionally, the primer RSS13 (5'-CCATGCTCATGCTGGATCTTTCC-3') was used for sequencing the 5' end of *PRP44*, including the His and Flag tags and the promoter region. Oligo-mediated site-directed mutagenesis was carried out using the Quick-Change™ site-directed mutagenesis kit from Stratagene. Four different pairs of primers were utilized to mutate the GKT and DExH domains into DNT and GExH respectively. DN1 (5'-CCACTGGTTCAGATAACACGAATATCGC-3') and DN2 (5'-GCGATATTCGTGTATCTGAACAGTGG-3') were used to create the DNT change in the first domain and DN3 (5'-GAAAGGGCACAGATAATACAGCTATGGC-3') and DN4 (5'-GCCATAGCTGTGTATCTGTGCCCTTTC-3') were used to mutagenize the GExH domain. DNI (5'-GAAAGGGCACAG ATAATACAGCTATGGCTG-3'), DNII (5'-CAGCCATAGCTGT ATATTCTGTGCCCTTTC-3'), DNIII (5'-TAATGATATATGAA GACGCTCATGAGATTA-3') and DNIV (5'-TAATCTCATGAG CGTCTTCATATATCATTA-3') were utilized to create the analogous mutations in the second putative helicase domain. Following the mutagenesis procedure, mutant clones were identified by DNA sequence analyses. Each mutagenized DNA plasmid was transformed into a wild-type cell, 1827, and streaked onto plates with or without 1 mM copper to monitor the effect of induction. The four oligos utilized for sequencing were as follows: 1st DNT: 5'-TTTCACGCAGCATTTGAAG GTG-3', 1st GExH: 5'-ACGCCAGAGAAATGGGAC-3', 2nd DNT: 5'-CAGAGCCAGGTCTTCGAG-3', and 2nd GExH: 5'-TCACGTCGTTGGAGACAA-3'. The *PRP44* coding region was also cloned downstream of the *GAL1* promoter by insertion into the *KpnI* site of the yeast vector pYES2.0 from Invitrogen (San Diego, California). This was accomplished by use of the following two PCR primers (RSS14: 5'-

TAAGGTGAAGGTACCTATAAGTAATTG-3' and RSS15: 5'-TGCTATAAACTGTAGGTACCACAATTAGATTA-3') and the *Pfu* PCR system from Stratagene. The cloned gene was transformed into a *Ts* allele of *PRP44* and tested at 36 °C.

Total yeast genomic DNA was used with the *Pfu* PCR system (Stratagene) to clone the wild-type *PRP16* and *PRP22* genes. For cloning of *PRP16*, two primers were used (16-1: 5'-TAAACACAAGAATTCCCAAAGACAATACA-3' and 16-2: 5'-AACTAAAAAAGAATTCCTTCTTTTGGAA-3'). The PCR products were digested with *Eco*RI and gel purified. The products were ligated into the *Eco*RI site of pYES2.0 vector (Invitrogen). The following primers were used to clone the *PRP22* gene (22-1: 5'-CTAATTTTCAACATAGGTACCACAAA-3' and 22-2: 5'-ATAGGTCTATAAATCTAGATAATTAT-3'). The resultant PCR products were digested with *Kpn*I and *Xba*I and gel purified. *Kpn*I and *Xba*I digested pYES2.0 vector was used for cloning the *PRP22* gene PCR products. After cloning, the vectors harboring *PRP16* and *PRP22* were transformed into the *prp16-1* or *prp22-1* mutant strains to test for complementation at 36 °C.

The GKT residues in ATPase motif I of *PRP16*, *PRP22*, and *PRP44* were changed into DNT using the Quick Change™ site-directed mutagenesis kit from Stratagene, according to the manufacturer's recommended protocol. Primers 16-dn-1 and 16-dn-2 were used for mutagenizing *PRP16*, and primers 22-dn-1 and 22-dn-2 for *PRP22* (16-dn-1: 5'-GTGAAACGGGCTCAGATAATACCA-3', 16-dn-2: 5'-TGGTATTATCTGAGCCCGTTTCAC-3', 22-dn-1: 5'-ACAGGTTCCGATAATACCACTCAAA-3', 22-dn-2: 5'-TTTGAGTGGTATTATCCGAACCTGT-3'). Primers 16-s and 22-s were used to sequence each mutagenized region (16-s: 5'-TTAAACATAAGAGCAA CTG-3', 22-s: 5'-GTTGAAAAACCAGCTGATTG-3').

In vitro splicing and splicing inhibition experiments

The splicing extracts were prepared by a liquid-nitrogen method (Umen & Guthrie, 1995). Glass-bead extracts were prepared prior to or following induction with galactose. Overnight B4-7-2D cell cultures were washed and diluted by two-fold with fresh URA⁻ or YPD media depending on the purpose of the experiment. For copper induction 5 mM copper were added to the cells and incubated for an additional 4 h. The cell pellets were frozen at -80 °C after copper or galactose induction. Frozen cells were thawed and washed with pre-chilled buffer A (10 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) containing four proteinase inhibitors (1 mM phenazine methylene sulfanyl fluoride (PMSF), 1 mM Benzamidin acid, 1 μM Leupeptin, and 1 μM Pepstatin A). The cell pellets were resuspended in 150 μL of buffer A immediately before the extract preparation. An equal volume of glass beads (0.45 mm, from Sigma, St. Louis, Missouri) was added, and cells were broken by vortexing three times for 1 min interspersed with a 1-min chilling in ice. After a 1-min microfuge spin the supernatants were collected, a 1/10-vol of 2 M KCl was added followed by a 15-min microfuge spin at 4 °C. After the centrifugation step, the supernatants were collected and used directly in the splicing inhibition assays.

The standard splicing reaction has been described elsewhere (Kim & Lin, 1993). For testing of the splicing inhibition activity of *PRP44* mutant proteins, 4 μL of wild-type splicing

extract were mixed with 2.5 μL of the glass-bead extract described above. The mixtures were incubated for 15 min in ice. Following this incubation, 2 mM ATP, 2.5 mM MgCl₂, 3% PEG 8000, 60 mM potassium phosphate (pH 7.0), and 5 nM ³²P-labeled actin pre-mRNA transcript were added and the mixture was incubated at 23 °C for 30 min. For the splicing inhibition experiments with purified proteins, 10 ng of each protein were mixed with wild-type extract, and incubated for 15 min at 4 °C followed by addition of splicing substrate and the components described above.

Purification of *PRP44* protein and ATPase assay

The N-terminal region of *PRP44* was modified to encode six continuous histidine residues and a Flag tag. The tagged protein was induced using copper induction from the *CUP1* promoter (Trotta et al., 1997). Affinity purification of protein was carried out using yeast strain B4-7-2D grown at 30 °C. Five hundred microliters of saturated culture were washed two times with sterile water and resuspended into 2 L of fresh media. The culture was incubated for an additional 5 h before induction with 5 mM copper for 7 h at 30 °C. Cell pellets were resuspended in 20 mL of buffer A containing a mixture of the four protease inhibitors described above and used for liquid nitrogen extract preparations (Umen & Guthrie, 1995). For purification of the His-tagged *PRP44* protein, the extract was mixed with an equal volume of 1× nickel column binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9) and subsequently applied to a nickel-chelating column from Novagen according to instructions supplied by the manufacturer. The His:Bind resin was washed successively with 10 mL each of 1× charging buffer (50 mM NiSO₄) and 1× washing buffer (60 mM imidazole, 50 mM NaCl, and 20 mM Tris-HCl, pH 7.9), and the protein-containing sample was then applied to the column. The columns were then washed with 20 mL each of 1× binding buffer and 1× washing buffer by free flow. The His-tagged *PRP44* proteins were eluted with successive washes of 10 mL of elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). The Prp44p-containing solution was concentrated using a Centricon 30 filtration device (Amicon, Beverly, Massachusetts) with a 1,000 × g spin for 30 min. The protein was resuspended in 10 mL of buffer BP (0.5 mL EDTA, 1 mM PMSF, 0.01% NP-40, 10% glycerol, 5 mM DTT, 50 mM KCl, and 20 mM HEPES, pH 7.7).

The *PRP44* proteins were further purified by anti-Flag antibody-mediated coimmunoprecipitation. This was accomplished by preincubating 100 μL of anti-Flag antiserum (Sigma) with protein A-Sepharose CL 4B beads (50 μL of 1 g protein A-Sepharose CL-4B per 10 mL, from Sigma) in 1 mL IPP-500 (10 mM Tris, pH 8.0, 500 mM NaCl, and 0.1% NP 40) at 4 °C overnight (Schwer & Guthrie, 1992b). The beads were washed in 5 mL of IPP-500 and 5 mL of IPP-150 (IPP buffer with 150 mM NaCl). The beads were mixed with the extracts in buffer BP and incubated in a cold room for 5 h. The beads were washed three times with 20 mL of ice cold IPP-150. The proteins in washed beads were eluted by successive incubations with 20 μL of 1 mg/mL Flag peptide (Sigma) and two times with 500 μL of 1 mg/mL Flag peptide in IPP-150 buffer at 23 °C for 10 min (Trotta et al., 1997). Each eluate was dialyzed two times against 2 L of buffer D (20 mM HEPES-

KOH, pH 7.8, 0.2 mM EDTA, 50 mM KCl, 20% glycerol, and 0.5 mM PMSF) at 4 °C for 2 h. The eluted *PRP44* proteins were tested for purity by Western blotting and silver staining (Sambrook et al., 1989).

The purified *PRP44* proteins were assayed for ATP hydrolysis in a 20 μ L reaction mixture containing 20 mM HEPES-KOH (pH 7.4), 2 mM DTT, 5 mM EDTA, 50 mM KCl, 100 μ M BSA, 100 μ M unlabeled ATP, 1 μ Ci of [γ - 32 P]-ATP (4,500 Ci/mM), and 0.5 μ g/ μ L of poly(U), containing 20 ng of each protein (Kim & Lin, 1993). The reactions were mixed and incubated at 23 °C. Hydrolyzed 32 P in the supernatant were measured by Cerenkov counting. The background resulting from incubation in the absence of the protein was subtracted from each value.

RNA preparation and quantitative detection of snRNAs

Saturated B4-7-2D cell cultures were pelleted, washed with sterile water, and diluted twofold by resuspending in fresh media. The cultures were grown at 30 °C for an additional 4 h in this media. Cells were then washed two times with sterile water, resuspended in the same volume of media, incubated for 4 h in the presence or absence of copper to see the effect of protein induction. Following the incubations, 5–10 mL of culture were used to prepare total cellular RNA using the RNeasy prep kit from Qiagen.

For the effect of the wild-type *PRP44* protein on relieving the accumulation of U4/U6 duplex, three different RNA samples were prepared. The wild-type cell, 1827, was transformed with two different plasmids, one with wild-type *PRP44* under the *GAL1* promoter and the other with wild-type or a dominant negative mutant under the *CUP1* promoter (for details, see legend to Fig. 6A). Two different strains were grown to saturation in 15 mL of LEU⁻/URA⁻/glucose media. After being washed with sterile water, the cells were resuspended in 30 mL of fresh media and grown for 4 h. Ten milliliters of culture were withdrawn and incubated for an additional 4 h and frozen at -80 °C. For induction of the *CUP1* promoter, 20 mL of culture were washed and incubated in URA⁻/LEU⁻/glucose media in the presence of 5 mM copper. After 4 h induction, 10 mL of culture were withdrawn and frozen at -80 °C until RNA was prepared. The last 10 mL of culture were pelleted, washed with sterile water, and incubated in URA⁻/LEU⁻/galactose media in the absence of copper to induce the wild-type Prp44p.

To prepare total cellular RNA, the cell pellets were mixed with an equal volume of glass beads (0.45 mm from Sigma) and vortexed vigorously for 1 min followed by cooling in ice for 1 min. This process was repeated twice. For native RNA hybridization analyses, the following oligodeoxynucleotide primers were used. The oligo U6-end (5'-AAAACGAAATAA ATCTCTTTGTAA-3'), oligo U4-end (5'-AAAGGTATTCCTAA AATTCCCTA-3'), oligo U5 (5'-AAGTTCCAAAAATATGG CAAGC-3'), oligo U1 (5'-CCGTATGTGTGTGTGACC-3'), and oligo U2 (5'-AAGAACAGATACTACACT-3') were used for U6, U4, U5, U1, and U2 snRNA detection, respectively. The oligos were kinase labeled with 32 P and gel purified before use. For native RNA hybridization, 2.5 μ g of total RNA were mixed with 5 nM of 32 P-labeled primers and vacuum dried without heat. The dried RNA and probe mixture were dissolved in 6 μ L of hybridization buffer and incubated at 37 °C for 15 min

(Li & Brow, 1993). The reactions were stopped by chilling in ice, mixed with 6 μ L of loading dye, and electrophoresed in a 9% nondenaturing polyacrylamide gel with 1 $\times$ TBE buffer at 175 V in a cold room (Li & Brow, 1993).

For the U4/U6 duplex unwinding activity of *PRP44*, RNA samples were prepared as follows. Wild-type cells were transformed with a vector harboring either a dominant negative mutant or wild-type *PRP44* under the *CUP1* promoter, and with the vector containing wild-type *PRP44* coding region under the control of the *GAL1* promoter (also see Fig. 6A). Thirty milliliters of cells were grown in glucose media without copper. From this culture, 10 mL were withdrawn and frozen at -80 °C for future RNA preparation. The remaining 20 mL of cells were washed and resuspended in media containing 5 mM of CuSO₄ and incubated for 4 h, at which time 10 mL were withdrawn and stored at -80 °C. The final 10 mL of cells were washed and resuspended in galactose media without copper for 5 h incubation to induce wild-type Prp44p.

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